Disentangling variational bias: the roles of development, mutation and selection

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Abstract

The extraordinary diversity and adaptive fit of organisms to their environment depends fundamentally on the availability of variation. While many evolutionary studies assume that random mutations produce isotropic phenotypic variation, the distribution of variation available to natural selection is more restricted, as the distribution of phenotypic variation is affected by a range of factors in developmental systems. Here, we revisit the concept of developmental bias – the observation that the generation of phenotypic variation is biased due to the structure, character, composition, or dynamics of the developmental system – and argue that a more rigorous investigation into the role of developmental bias in the genotype-to-phenotype map will produce fundamental insights into evolutionary processes, with potentially important consequences on the relation between micro- and macroevolution. We discuss the hierarchical relationships between different types of variational biases, including mutation bias and developmental bias, and their roles in shaping the realized phenotypic space. Furthermore, we highlight the challenges in studying variational bias and propose potential approaches to probe developmental bias.

Introduction

The observed diversity of life on Earth represents a fraction of theoretically possible phenotypes in living organisms (Lewontin, 1979; Alberch, 1989, 1980; McGhee, 2006). Indeed, the fossil records demonstrate prolonged periods of stasis in numerous lineages, and convergent evolution is widespread (Allen *et al.*, 2008; Alberch, 1985). Yet, an enduring fundamental assumption of modern evolutionary genetics is that "universal variability – small in amount but in every direction" is a key factor governing the agency of natural selection (Wallace, 1871; Hine *et al.*, 2014). For example, Fisher's geometric model assumes that mutations are isotropic, i.e., the magnitude and direction of effects prior to selection are random in phenotypic space (Fisher, 1999; Tenaillon, 2014). This assumption remains a dominant paradigm in population genetics, despite mounting evidence challenging its universality (Wright, 1984; Wagner & Zhang, 2011). Phenotypic variation is profoundly structured, and variation and evolution are related at multiple timescales (Melo *et al.*, 2016; Houle *et al.*, 2017; Holstad *et al.*, 2024). While functional and population geneticists have made great progress towards an understanding of heredity, mapping genotype onto phenotype, and the mechanisms and consequences of natural selection, the structure of variation accessible to selection remains elusive (Arthur, 2010). The structure of variability leads to variational constraints, defined by limitation and biases in the variability of phenotypic characters, which is one of the central classes of evolutionary constraint (Pigliucci & Preston, 2004). These constraints are the outcome of a broader set of factors, including genetic architecture, mutation, development, and are observed at all levels of biological organization (Fig. 1). Different types of constraints can affect evolutionary processes – and phenotypic outcomes – in distinct ways. It is thus critical to clarify what we measure when we measure variational constraints, and how different types of variational bias relate to one another, as they are frequently intertwined.

Identifying the sources of variational bias

Variational constraints are routinely characterized by measuring the linear association between traits in a population, and covariance matrices derived from these phenotypic measurements will capture different aspects related to the causes and consequences of variational bias(Arnold *et al.*, 2008; Steppan *et al.*, 2002; Henry & Stinchcombe, 2023; Houle *et al.*, 2017; Falconer *et al.*, 1996; Penna *et al.*, 2017; Melo *et al.*, 2019). For example, genetic information summarized by the additive genetic variance-covariance matrix – commonly referred to as $\mathbf{G}$ – is a common measure of variational constraint in plant and animal breeding. $\mathbf{G}$ specifically describes trait covariance due to pleiotropic

alleles, wherein variation at a single locus has effects on multiple traits, or due to linkage disequilibrium of two loci that are strongly associated in populations (Lande, 1980; Lynch *et al.*, 1998; Falconer *et al.*, 1996; Conner *et al.*, 2004). In evolutionary quantitative genetics, **G** represents an important constraint because it describes the degree to which the genetic architecture (i.e., how traits are genetically connected to each other) may determine the response of a population to selection (Schluter, 1996; Lande & Arnold, 1983). Indeed, the genetic variation or **G** is the most relevant parameter to the concept of "evolvability", viewed as the population's capacity to respond to directional selection (Jones *et al.*, 2007; Hansen & Houle, 2008). The context-dependency of **G**, the evolution of evolvability itself, and how evolvability predicts the trait divergence and stasis under selection has garnered much of the empirical and theoretical attention to variational bias over recent decades (Wood & Brodie III, 2015; Opedal *et al.*, 2023; Walter, 2023; Pujol *et al.*, 2018; Mallard *et al.*, 2023a,c; Le Rouzic *et al.*, 2013; Hansen & Wagner, 2023; Walsh & Blows, 2009; Walter & McGuigan, 2023; Arnold *et al.*, 2008; Chenoweth *et al.*, 2010; Johansson *et al.*, 2021; Voje *et al.*, 2023; Holstad *et al.*, 2024).

While **G** is a measure of the amount and structure of standing genetic variation, new variation generated by new mutations can be characterized by a mutational variance–covariance matrix **M** (Dugand *et al.*, 2021; Houle *et al.*, 2017). The **M**-matrix can be estimated through mutation accumulation (MA) experiments under a relatively selective-neutral environment. While **M** itself provides information about the Genotype-to-Phenotype map, and hence developmental bias, it also captures bias caused by heterogeneous mutation rates and mutation spectra across the genome (Fig. 1)(James *et al.*, 2023; Rohner & Berger, 2023; Sane *et al.*, 2023; Cano *et al.*, 2023; Yampolsky & Stoltzfus, 2001; Katju & Bergthorsson, 2019; Agashe *et al.*, 2023; Couce & Tenaillon, 2019). In other words, **M** can reflect the inherent limitations in the genotype space that favor specific mutational outcomes (e.g., more mutable single nucleotide, transition-transversion bias). Thus, **M** acquired through mutation accumulation experiment includes developmental bias, but not limited to developmental bias (Rohner & Berger, 2023). Mutation bias is the bias specifically produced during the mutational process (Fig. 2 Right) without including the effects of development and selection on phenotypes (Fig. 2 Middle). Thus, **M**, the mutation variance-covariance matrix, naturally captures both developmental and mutation bias.

The magnitude and direction of **G** can provide information about the degree to which evolutionary constraints may be present in a population. **G** is the product of many factors, including development, mutation, selection, drift, migration, and inbreeding. (Guillaume & Whitlock, 2007; Chebib

& Guillaume, 2017; Chantepie & Chevin, 2020; Phillips *et al.*, 2001; Cai *et al.*, 2023) The specific mechanisms and relative contributions of various factors in shaping and maintaining **G** remain poorly understood. A major complication is that, almost certainly, these contributions can drastically differ between different suite of traits or trait combinations. Empirically, the contributions of **M** and selection to shaping **G** can be inferred by comparing **G** to **M**, or comparing **G** to γ, the matrix describing multivariate nonlinear selection. If **G** is shaped by **M**, then, in equilibrium, **G** should be proportional to **M** (Cheverud, 1984). A recent simultaneous estimate of both **G** and **M** in the same *Drosophila serrata* population indeed shows some proportionality between the two matrices, showing a contribution of mutation bias to additive genetic variation (Dugand *et al.*, 2021). Surprisingly, in this population, **M** appears to be more constrained than **G**, which implies that selection can act to break constraints imposed by mutation, instead of the usual picture of stabilizing selection increasing genetic correlations. This result illustrates how nonintuitive and case dependent the shaping of **G** might be. Furthermore, other multivariate analyses suggest differences between **G** and **M** (Latimer *et al.*, 2011; Houle *et al.*, 1994; Keightley *et al.*, 2000; Latimer *et al.*, 2014; Camara *et al.*, 2000; Mallard *et al.*, 2023b). Notably,Houle *et al.* 2017 found **G** and **M** to be markedly similar for wing traits in *Drosophila melanogaster*. Moreover, **M** reliably predicts patterns of wing divergence across drosophilids(Houle *et al.*, 2017), suggesting a major role for mutation in determining long-term evolutionary divergence, as well as **G**.

One distinction between **G** and other variational constraint is that developmental and mutational biases can vary among individuals and genotypes both empirically and in theory, (Psujek & Beer, 2008; Uller *et al.*, 2018; Braendle *et al.*, 2010; Mallard *et al.*, 2023b; Conradsen *et al.*, 2022) while **G**, or more broadly phenotypic correlation, is a measure of a given population. The (co)-variance in **G** explained by each polymorphic locus is affected by allele frequencies and the magnitude of allelic effects in an individual (Benfey & Mitchell-Olds, 2008; Kelly, 2009).

Theoretical and empirical studies suggest that **M**-induced genetic correlations tend to be more stable than genetic correlation caused by selection, implying that identifying the mechanisms causing genetic correlation may help us understand the evolution of **G** (Jones *et al.*, 2003; Cai *et al.*, 2023). Furthermore, the genetic architecture of **G** may be informative in inferring the drivers of **G** (selection versus **M**) since those structures of linkage disequilibrium and pleiotropy in the genome are footprints of distinct forces when inducing and maintaining **G**. Selection can also reshape **M** (Fig. 2), although the timescale on which the evolution of **M** occurs – relative to phenotypic evolution – is unclear; we may be able to treat **M** as constant under most evolutionary scenarios (Mallard *et al.*,

2023b; Conradsen *et al.*, 2022).

Collectively, we argue that clear and distinctive definitions of variational biases at different levels are needed to ensure effective communications and nuanced analyses in the future (Fig. 1).

Developmental bias in the production of phenotype

An important aspect of genotype-to-phenotype maps is that they are highly non-linear and structured in nature. Therefore, random mutations do not necessarily produce random phenotypic changes. The distribution of phenotypic variants that occur as a result of genetic and environmental variation is channelled by the developmental processes that transform the embryonic phenotype into the adult form (Klingenberg, 2008; Snell-Rood & Ehlman, 2023). This developmental process imposes a bias on the generation of phenotypic variation, arising from the structure, character, composition, or dynamics of development, relative to the assumption of isotropic variation, resulting in developmental bias (Maynard Smith *et al.*, 1985; Sears, 2014; Uller *et al.*, 2018; Alberch, 1989).

A number of empirical studies have hypothesized that an organism's developmental system shapes the trait-trait (co)variance observed in **G**, which has been described as phenotypic integration (Pigliucci & Preston, 2004). Development is therefore a critical factor in shaping the variational bias reflected in **M** and **G** (Hallgrímsson *et al.*, 2009). Substantial evidence has shown that this developmental bias is common (Staps *et al.*, 2023; Machado *et al.*, 2023; Couzens *et al.*, 2021; Rohner & Berger, 2023; Staps *et al.*, 2023). For example, the developmental regulation of tetrapod limb generates bias in the number and distribution of digits and limbs (Alberch & Gale, 1985; Wake, 1991); Interactions among components in a developmental system bias trait-trait relationships in insects and pigment coloration of insect wings (Brakefield & Roskam, 2006; Prud'homme *et al.*, 2006). While selection ultimately shapes developmental systems, such intrinsic biases from developmental systems are likely to be an important component of phenotypic evolution (Wagner & Altenberg, 1996; Sears, 2014; Uller *et al.*, 2018; Moczek *et al.*, 2015; Staps *et al.*, 2023). Firstly, the bias in genotype and phenotype production stands as a distinct phenomenon from phenotypic adaptation, each subject to separate evolutionary dynamics (Wagner & Altenberg, 1996; Watson *et al.*, 2014). Conventionally, developmental biases are believed to undergo more gradual evolution in comparison to the traits that they influence (Watson *et al.*, 2014). Secondly, although certain parts of developmental systems remain evolvable and susceptible to selective pressures, prevailing global constraints resist alteration (Maynard Smith *et al.*, 1985; Brakefield, 2006). Exemplifying this notion, resource acquisition is limited due to chemical, thermodynamic, and mechanistic constraints (Novak *et al.*, 2006; Lipson,

2015; Reding-Roman *et al.*, 2017), leading to trade-offs between, e.g., growth rate and yield in *E.coli* (Novak *et al.*, 2006), or between spore number and quality in *D. discoideum* (Wolf *et al.*, 2015). Another famous example comes from the metabolic scaling law, which states that metabolic rate scales with body mass to the power of 3/4. A wide range of organisms over several orders of magnitude in body mass conform to this law. Of course, one could argue that it is purely natural selection that forces these points to fall along a predictable trajectory across evolutionary history. But there are theoretical arguments that demonstrate such metabolic scaling is caused, at least in part, by physical forces imposing constraints, or by biases in patterns of energy allocation (White *et al.*, 2022; Kooijman, 2010; West *et al.*, 2001).

Importantly, the relationship between adaptation of traits and developmental bias is not simply one of opposition, but is instead the result of a continuous dynamic interaction. Therefore, we can only ask whether developmental bias alters evolutionary trajectories in relatively short timescales (Pigliucci & Preston, 2004). For instance, two regulatory networks may yield similar functional outputs but differ in their variational properties, leading to different evolutionary biases (Schaerli *et al.*, 2018). However, over a timescale of macroevolution, it is difficult to disentangle the contribution of developmental bias and selection in phenotypic adaptation. Because firstly, bias of phenotypic production can evolve. Secondly, the evolutionary history of variational bias cannot be easily reconstructed unless the mutation and developmental bias remain relatively constant over macroevolutionary timescales. An outstanding practical problem is: can we treat developmental bias as constant, and at what timescales (Fig. 2).

Emerging methods for measuring developmental bias

The evolutionary significance of developmental bias has long been controversial because, we argue, it can be difficult to accurately diagnose. Natural selection and random genetic drift strongly affect the patterns of phenotypic variation within and between populations, making it unsatisfying to rely solely on measurement of existing phenotypic variation when attempting to identify developmental bias (Lynch *et al.*, 1998; Roseman, 2020). Given that both developmental bias and selection could create similar phenotypic distribution in natural populations, it is generally difficult to distinguish between the two (Schluter, 1996; Pigliucci & Preston, 2004). To quantitatively investigate developmental bias, researchers need to assess the propensity of phenotypic production prior to selection rather than merely observing the current state of variation (Wagner & Altenberg, 1996).

One traditional approach to distinguish between the effects of developmental bias and selection is

through mutation accumulation (MA) lines to assess the spectrum of phenotypic variation generated by *de novo* mutation in the absence of selection (Zalts & Yanai, 2017; Braendle *et al.*, 2010; Houle *et al.*, 2017). However, as mentioned above, *de novo* mutation captures not only the propensity of the developmental system to vary but also heterogeneity in mutational rates and spectra across genome, which constrains the mutation in genotype space (Stoltzfus & McCandlish 2017; Rohner & Berger 2023; James *et al.* 2023; Agashe *et al.* 2023; Sane *et al.* 2023; Fig. 1, e.g., more mutable single nucleotide, transition-transversion bias, etc.) Alternatively, some well-studied developmental systems, such as tooth morphology, can be modeled sufficiently well so that a large number of perturbations can be simulated to evaluate the variability *in silico* (Salazar-Ciudad & Jernvall, 2010). However, this method is only feasible for few systems for which we have a relatively complete knowledge of intricate developmental dynamics.

Another approach to establish developmental bias is to measure symmetry of the left and right sides of the same organism – so-called "fluctuating asymmetry" – (Rohner & Berger, 2023; Klingenberg & McIntyre, 1998), which share both a genome and environment. However, the developmental process may produce asymmetry in morphological traits due to inevitable consequences of molecular stochasticity, which is often interpreted as developmental noise. A recent study showed that developmental bias quantified using such internal variability in the dipteran wing predicts its evolution on both short and long evolutionary timescales (Rohner & Berger, 2023). Ultimately, addressing the of evolutionary role of developmental bias requires studies in more systems. We thus ask alternative and existing ways to characterize developmental bias without being dependent on specific developmental systems.

In line with the concept of fluctuating asymmetry, there are multiple ways to assess the propensity of the system to vary by inducing mild and random (environmental or genetic) perturbations. The phenotypic variation in a genetically identical population, under the same environmental condition, has often been referred to as intra-genotypic variability (Metcalf & Ayroles, 2020; Bradshaw, 1965) or phenotypic variability (Abley *et al.*, 2021; Ayroles *et al.*, 2015; Willmore *et al.*, 2007), which is thought to be an emergent by-product of the developmental processes (Willmore *et al.*, 2007). Such variability reflects both the results of stochasticity in molecular interactions and of external sources caused by microenvironmental variation (Elowitz *et al.*, 2002; Sanchez & Golding, 2013; Cortijo *et al.*, 2019). These extrinsic and intrinsic small random fluctuations interact with developmental systems and give rise to the phenotypic variability. Thus, we hypothesize that the variational properties induced by random small perturbations most likely reflect the inherent attributes of the system

rather than a certain direction of perturbation (e.g., changes of a nutrient level, single gene knock-out etc.). In fact, such phenotypic variability has been used to characterize developmental systems in many studies (Kiskowski *et al.*, 2019; Klingenberg, 2019; Geiler-Samerotte *et al.*, 2020), though these studies have not explicitly addressed developmental bias. For example, variational properties under the same environmental condition across clonal cells help to quantify inherent relationships among yeast morphology traits (Geiler-Samerotte *et al.*, 2020).

Over the last few decades, studies of gene expression evolution have proliferated owing to reduced cost of RNA sequencing. A surge of studies in canalization, modularity, and phenotypic integration at the molecular level has followed (Lea *et al.*, 2019; Cai & Des Marais, 2023; Melo *et al.*, 2023; Gibson, 2009). These concepts are all connected to the classic notion of developmental constraint (Melo *et al.*, 2016). Yet, few studies used expression variability to address these questions: much efforts in gene expression variability have been taken to examine the genomic, epigenetic, and topological features in determining the gene-specific expression variability level (Cortijo *et al.*, 2019; Barroso *et al.*, 2018). We argue that genome-scale expression variability data can be exploited to investigate the bias in the production of gene expression and, ultimately, contribute to our understandings of evolution in gene expression (Wolf *et al.*, 2023).

Another way to impose random perturbations is through random mutation. As discussed above, the variants captured in mutation accumulation experiments account for the heterogeneity of mutation rates and other mutational bias across the genome (Fig. 1). Unfortunately, MA studies provide a very limited view of the distribution of mutational effects because the number of spontaneous mutations sampled in each study tends to be very low (Hodgins-Davis *et al.*, 2019). One approach to ameliorate these limitations is to introduce mutation without incurring mutational bias during *de novo* mutation. For example, genome-wide mutagenesis (Hodgins-Davis *et al.*, 2019) as opposed to *de novo* mutation provides an empirical investigation of bias in the phenotypic production and reveal greater neutral expression divergence than commonly used models of phenotypic evolution. Deep-sequencing techniques can also be used to trace individual allele effects for single nucleotide variants across the genome (Dolan *et al.*, 2021; Gitschlag *et al.*, 2023). A similar outcome and mutational landscape of a given trait (trait combination) from wide-range variants across the genome would be indicative of developmental or mutation bias. Alternatively, artificial recombinant populations provide mutational perturbative materials for examining the propensity of the system to vary (Cai *et al.*, 2023; Fraser, 2020). In addition, systematic perturbation studies using the CRISPR-Cas9 methodology with the scale of massive high-throughput phenotyping or RNA-sequencing (e.g., perturb-seq (Dixit *et al.*,

2016)) can be leveraged to examine the variational properties of organisms.

Concluding remarks

One long-standing suggestion for bridging the gap between micro- and macroevolution has been through the study of evolutionary developmental biology and ontogeny (Machado *et al.*, 2023; Rolland *et al.*, 2023). Here, we argue that progress in integrating micro- and macro-evolutionary theory has been hampered by the common assumption in population genetics that genotype-to-phenotype mapping is a straightforward exercise emerging from an invariant distribution of mutational effects. There is substantial evidence that variational bias is common. Such bias may be caused by factors in mutation, genetics, and development. We argue that clear and distinctive definitions of variational biases at such different levels (Fig. 1 and Fig. 2) are needed to help better understand the role of variational bias in adaptation and how evolution shapes variational bias. Furthermore, as we have shown, developmental bias is notoriously difficult to establish empirically. We thus review and suggest approaches aimed at identifying developmental bias and testing for its role in shaping phenotypic evolution. In particular, we argue that a large number of untargeted and random perturbations can be exploited to assess the propensity of the system to vary, and hence, the bias of phenotypic production. Collectively, we present challenges in studying variational bias and its role in shaping evolutionary history and impacting future adaptation.

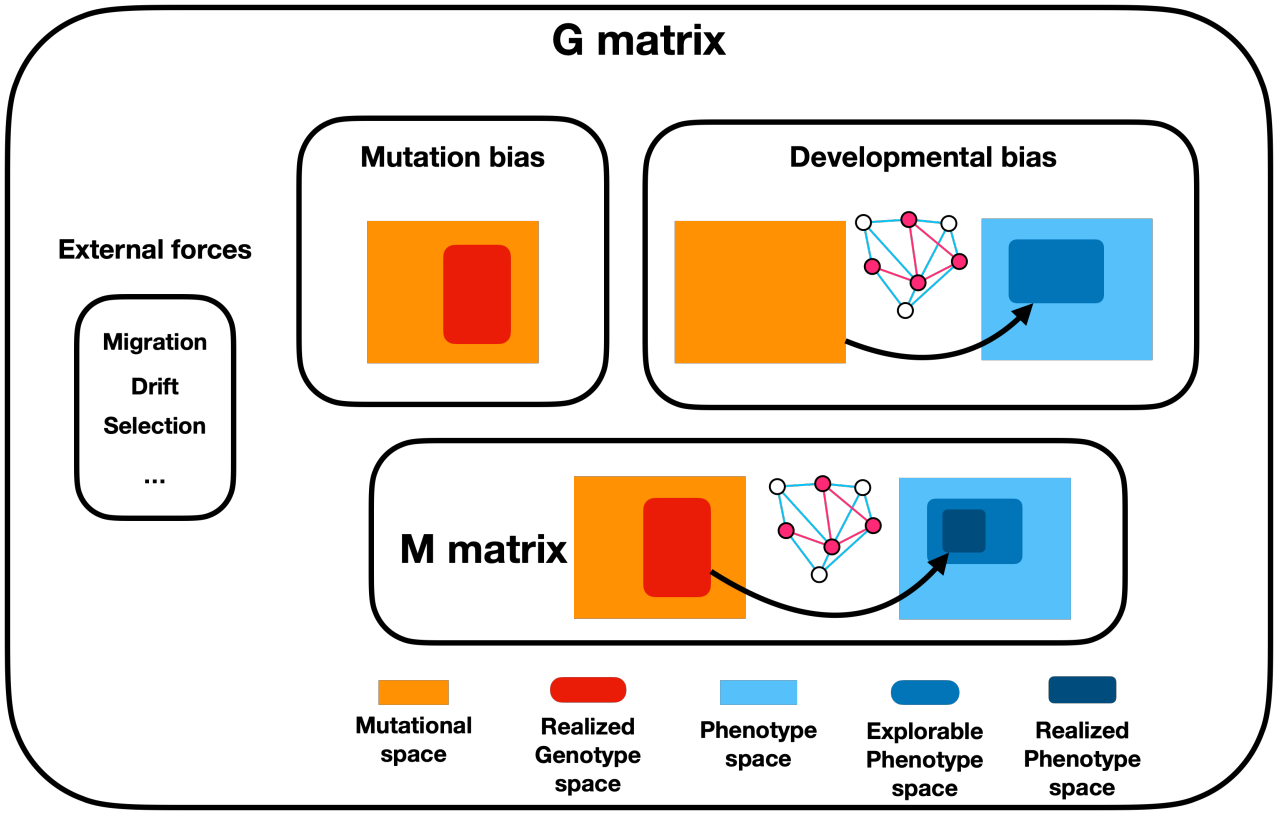

Figure 1: **Hierarchical relationships of the concepts and empirical measurements related to variational biases**. Because of mutational bias, only a subset of random mutation is realized in the genotype space of possible mutations. The genotype space is translated to the phenotype space through the process of development, which can impose developmental bias. Not all phenotypes in the phenotype space are accessible due to developmental bias, which leads to an explorable phenotype space that is a subset of the total possible phenotype space. Mutation accumulation (MA) lines captures both the mutation bias and developmental bias, which leads to a realized phenotypic space that is a subset of (explorable) phenotypic space. Therefore, the "explorable" phenotypic space is influenced solely by developmental bias while the "realized" phenotypic space is a result of both mutation and developmental bias as captured by mutation accumulation (MA) lines. Other evolutionary forces such as selection, migration, and drift interact with mutational variation (**M**) in the realized phenotype space to shape **G**.

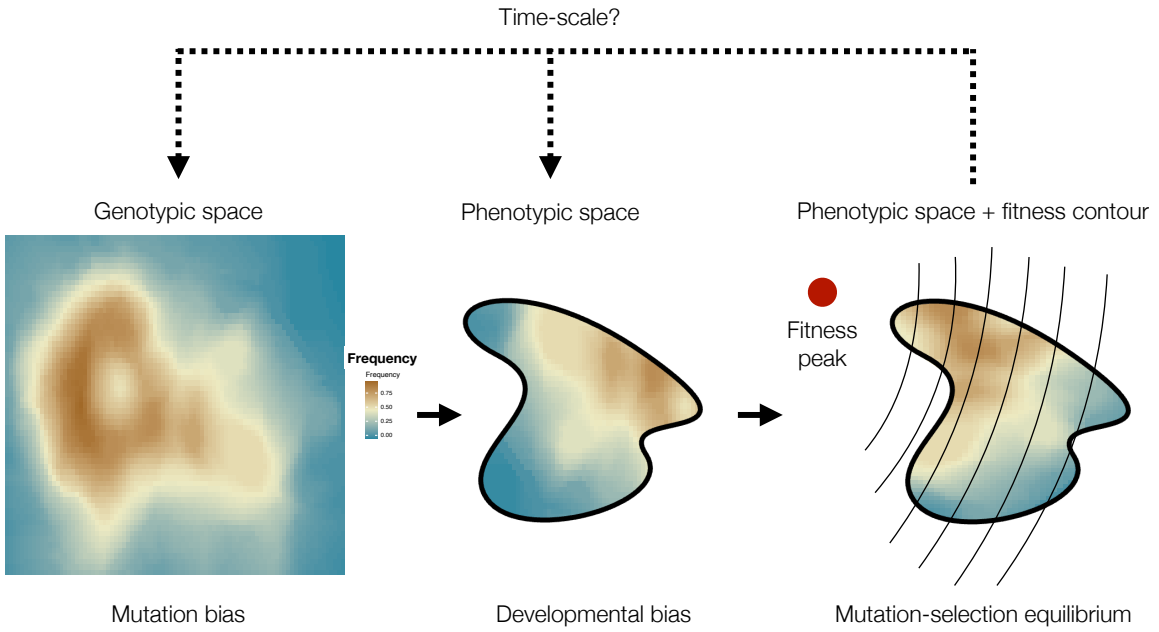

Figure 2: **A hypothetical scenario showing influences of mutation bias and developmental bias on adaptation**. Mutations are biased such that some mutations are more likely to occur than others (left). Developmental processes then translate genotypic variation into phenotypic space, potentially imposing developmental bias (middle). Such biased phenotypic distribution interacts with drift and selection to form the distribution of population under mutation-selection equilibrium (right). However, both mutation bias and developmental bias can evolve in response to selection. Whether the timescale of the evolution of developmental and mutation bias is longer than the trait adaptation is often unknown. Solid arrows represent processes occurring over short time scales (micro-evolution), while dashed arrows indicate processes that possibly occur over similar or longer evolutionary time scales (macro-evolution).

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